Aquatic Toxicology 75 (2005) 253–262

AQUATIC TOXICOLOGY

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Joint action of polycyclic aromatic hydrocarbons: Predictive modeling of sublethal toxicity

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Received 13 May 2005; received in revised form 17 August 2005; accepted 17 August 2005

Abstract

Polycyclic aromatic hydrocarbons (PAHs) typically contaminate the environment as complex assemblages of different chemical compounds. Modeling approaches provide a means of estimating the toxicity of these PAH mixtures. In the present study, we tested the hypothesis that the joint effects of four PAHs: pyrene, phenanthrene, fluoranthene and naphthalene, on the growth rate of the crustacean *Daphnia magna* during sub-chronic exposure could be accurately predicted using a mathematical algorithm for concentration addition based upon the assumption that these PAHs impact growth by a common mode of action. Assessment of the individual toxicity of the four PAHs confirmed that these compounds elicited the common effect of retarding growth of daphnids at concentrations below those that were lethal to the organisms. Using the experimentally derived toxicity parameters for the individual chemicals, the toxicity of multiple mixtures of these four PAHs was modeled. These mixtures were based on concentrations reported in the environment and on equi-toxic concentrations. The effects of over 140 combinations of four mixture formulations on the growth rate of daphnids were experimentally determined and compared to model predictions. The concentration addition models tended to over predict the joint toxicity of these PAH mixtures and experimental data was better represented by an alternative model based upon the concept of independent joint action. Mixtures at environmentally relevant concentrations were predicted and experimentally demonstrated to have no effect on daphnid growth rates. Results indicate that PAHs elicit toxicity to daphnids by multiple mechanisms and demonstrate an appropriate modeling approach to assess the toxicity of these mixtures.

Keywords: Hazard assessment; Risk assessment; Response addition; Synergy; Mixture toxicology

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are continually introduced into the environment through both natural and human activities including incomplete combustion from the burning of fossil fuels, forest fires and volcanic activity (Baek et al., 1991; ATSDR, 1995; Howsam and Jones, 1998). They may also enter aquatic systems from industrial and water treatment plants, seepage, and accidental spills (ATSDR, 1995). These chemicals have become nearly ubiquitous in freshwater rivers, streams and lakes. Of the 126 toxicants the

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doi:10.1016/j.aquatox.2005.08.007

US Environmental Protection Agency has designated as "priority toxic pollutants" under the guidance of the Clean Water Act, 16 are PAHs. Owing to the nature of their formation, however, they are never present as single chemical compounds, but rather exist in the environment as mixtures (ATSDR, 1995). The development and validation of predictive models for estimating the effects of exposure to PAH mixtures will enhance the risk assessment for these toxicants.

One methodology that has been used to predict the toxicity of defined mixtures in aquatic toxicology is concentration addition (Niederlehner et al., 1998; Altenburger et al., 2000; Cleuvers, 2003). Concentration addition is expressed mathematically as:

$$\sum_{i=1}^{n} \frac{C_i}{\mathrm{EC}x_i} = 1 \tag{1}$$

where C_i is the concentration of the ith constituent of a mixture of n chemicals and x is the effect of the whole mixture. $\mathrm{EC}x_i$ is the concentration of the ith constituent that elicits the same response as the mixture (x) when exposure occurs to that chemical alone. The underlying assumption of concentration addition models is that the chemicals composing the mixture elicit their effects via the same mode of action.

The toxic equivalency approach is derived from concentration addition when one further assumes that the individual chemicals within a mixture have concentration–response curves that are parallel to one another. This approach effectively normalizes the concentrations of chemicals to that of a reference chemical using the ratios of the EC50s (Safe, 1998). This can be expressed mathematically as:

$$C_{\mathrm{eff}} = \sum_{i=1}^{n} C_i \times \frac{\mathrm{EC50_r}}{\mathrm{EC50}_i} \tag{2}$$

where C_{eff} is the effective concentration of the mixture in terms of the reference chemical, r. Here, C_i represents the concentration of each chemical in the mixture and $\mathrm{EC50}_i$ represents the EC50 of the respective (ith) chemical. $\mathrm{EC50_r}$ is the EC50 of the reference chemical. This approach has been used extensively in the assessment of mixtures of dioxin-like chemicals, where the reference chemical used is TCDD (Kovacs et al., 1993; Newstead et al., 1995; Hahn et al., 1996; Zabel et al., 1996; Steevens et al., 2005). The toxic equivalency approach has also been applied to PAHs with respect to carcinogenicity (see review (Delistraty, 1998)), however, its applicability to PAH mixtures in aquatic toxicology is largely undetermined.

The alternative approach to predicting mixture response based on individual chemical parameters is that of independent joint action (Bliss, 1939; Backhaus et al., 2000) which is expressed mathematically as:

$$R_{\mathrm{mix}} = 1 - \prod_{i=1}^{n} (1 - R_i) \tag{3}$$

where R_{mix} is the total mixture's response and R_i is the response of chemical i. This method is based on probability theory where one is combining independent probabilities of an event occurring and then subtracting the overlap. In this approach, the underlying assumption is that the individual constituents each elicit their response through differing mechanisms of action.

In this study, we evaluated the effects of four PAHs, both individually and in mixtures, on growth rates of the water flea, *Daphnia magna*. Using values obtained from a recent survey of freshwater streams in the United States (Kolpin et al., 2002), we modeled and experimentally determined whether mixtures of these four chemicals would impact daphnid growth rates at environmentally relevant concentrations. We then evaluated the toxicity of equi-toxic mixtures of these PAHs in order to compare models of concentration addition and independent joint action for these PAHs. For all mixtures we hypothesized that joint action of these PAHs could be best modeled using a concentration addition approach due to a presumed common mode of action of the chemicals.

2. Materials and methods

2.1. Cultures

The line of daphnids used in these experiments has been cultured at North Carolina State University for over 10 years with culture conditions described previously (Baldwin and LeBlanc, 1994). Daphnids were cultured in 1 L of medium at a density of 40 animals per beaker with medium changes three times a week. Food

was provided twice daily in the amount of 1.4×10^8 cells of the green algae, *Selenastrum capricornutum*, and ~4 mg dry weight of a fish food homogenate (Tetrafin, Pet International, Chesterfill, NSW, Australia). Cultures were maintained in an environmental chamber with a 16:8 h light:dark photoperiod at 20 °C. Algae were cultured in Bold's Basal medium at room temperature and constant lighting. All experiments were begun with daphnids at an age of less than 24 h old.

2.2. Single PAH exposures

The effects of four PAHs listed as priority pollutants: pyrene (Fluka Chemika, Milano, Italy), naphthalene (Fluka Chemika), phenanthrene (Sigma–Aldrich, St. Louis, MI, USA) and fluoranthene (Sigma–Aldrich) on daphnid growth rates were characterized individually. The purity of each PAH was >99.0%. Each experiment consisted of 50 nominal concentrations of the PAH and 10 control treatments. PAHs were delivered to the aqueous media in ethanol. Solutions were replaced every other day. All treatments, including controls, contained <0.01% (v/v) ethanol and was constant in all treatments for a given experiment. Only one daphnid was exposed to each concentration, which was prepared at either 90 (fluoranthene, phenanthrene and naphthalene) or 95% (pyrene) of the next highest concentration. The highest concentration in each experiment was based on acute studies and chosen to approximate the lowest lethal concentration over the exposure period of 7 days. Each day, beakers were examined for the presence of a shed exoskeleton, which was removed and examined under magnification. The length of the exoskeleton was measured from the top of the carapace to the base of the tail spine with an ocular micrometer (4× or 10× magnification). For each daphnid, the lengths of the first four exoskeletons were plotted against the molt number and fitted with linear regression. The slope of this line represents the growth rate for the daphnid. This approach to measuring growth rates in daphnids is described in greater detail in previous work (Olmstead and LeBlanc, 2001). The mean of the growth rates from the control daphnids was taken as the normal growth rate and treated daphnid growth rates were expressed as the percentage growth rate reduction due to PAH exposure. Experimental data was plotted with Origin software (Microcal™ Software Inc., Northampton, MA, USA) and fitted with a sigmoidal concentration–response curve in the following form:

$$R = \frac{100}{1 + \left(\frac{\mathrm{EC50}}{C}\right)^{\rho}} \quad (4)$$

where R is the growth rate reduction expressed as a percentage, C the concentration of the PAH and EC50 and ρ are parameters of the concentration–response curve representing the concentration that elicits a 50% response and the power of that curve, respectively. These fitted parameters were then used in subsequent mixture modeling.

2.3. PAH mixture exposures

Mixture exposures were similar in design to the individual PAH toxicity characterization experiments. Each exposure was based on a defined mixture of PAH concentrations that was designated as the base level for a given exposure. Concentrations of the PAHs in the base levels of all the mixtures are presented in Table 1. Dilutions and fortifications of the mixtures were used as treatments, with each treatment consisting of a concentration 90% of the next highest treatment level. These were fixed ray type mixture exposures, where all treatments in an experiment consisted of PAH concentrations in the same ratio. Again, one daphnid was exposed to each treatment. Environmentally relevant mixtures were based on values reported in a recent survey of US streams (Kolpin et al., 2002). One mixture consisted of the PAHs in a ratio identical to the median detected concentrations reported, while another con-

Table 1
Base levels of PAHs for three mixture formulations examined

PAH	Mixture		
	Median (μg/L)	Maximum (μg/L)	EC05 (μg/L)
Pyrene	0.050	0.84	22
Phenanthrene	0.040	0.53	46
Fluoranthene	0.040	1.2	39
Naphthalene	0.020	0.080	690

Median and maximum values were obtained from a survey of freshwaters in the United States (Kolpin et al., 2002). EC05 values are based on the individual chemical toxicity evaluations and were calculated using Eq. (4).

sisted of a ratio of maximum detected concentrations of each PAH reported in the survey.

Further mixture experiments were performed to evaluate the appropriateness of using a toxic equivalency model versus an independent joint action model. One experiment had the same design as the environmental survey-based mixtures, using a base level where each individual PAH was present at its EC05 (Table 1). Each treatment level was set at 95% of the next highest treatment level. Another experiment examined a series of mixtures where treatments consisted of each of the four PAHs at equi-toxic concentrations. Equi-toxic mixtures of PAHs ranged from treatments consisting of all chemicals at their EC005–EC15 level. For this experiment 10 daphnids were individually exposed to each treatment and treatments were compared to the control with a Dunnett's *t*-test (Piegorsch and Bailer, 1997).

2.4. Mixture model

The predicted effects of the various mixtures were determined by combining Eqs. (2) and (4) yielding the following:

$$R_{\text{mix}} = \frac{100}{1 + \left(\frac{1}{\sum_{i=1}^{4} \frac{C_i}{\text{EC50}_i}} \right)^{\rho'}} \quad (5)$$

where R_{mix} is the response of the mixture, C_i and EC50_i the concentration and EC50 of a given PAH and ρ' is the average power of the concentration–response curves for the four PAHs. C_{eff} and EC50_{r} in Eq. (2) was inserted into Eq. (4). Since the EC50_{r} is cancelled out, it is not necessary to implicitly state a reference PAH for our mixtures (Olmstead and LeBlanc, 2005). The toxicity of each mixture was modeled for concentration additivity using Eq. (5) for the toxic equivalency approach and Eq. (1) for the general concentration addition model by iteratively solving for the mixture effect (x). Mixture toxicity also was modeled according to independent joint action (Eq. (3)). Model predictions and experimental results were compared using coefficients of determination (Zar, 1996).

3. Results

3.1. Single PAH exposures

Individual exposure to non-lethal levels of each of the PAHs resulted in reduced growth rates (Fig. 1). EC50 and ρ parameters for each PAH derived from these exposures are presented in Table 2. Pyrene and fluoranthene reduced growth rates at similar concentration ranges, ~10–100 μg/L. Phenanthrene reduced growth rates at ~40–400 μg/L. Naphthalene was less effective at retarding daphnid growth rates, eliciting effects at concentrations >1000 μg/L.

3.2. Mixtures

The effects of PAH mixtures, in which the ratio of the individual constituents was equivalent to the ratio of the chemicals when present at their median environmental levels, was modeled using the concentration addition approaches (Fig. 2A, toxic equivalency, solid line; general concentration addition, dotted line). Both models indicated that PAHs present at their median environmental levels (base level = 1, Fig. 2A) would elicit no adverse effect on growth of daphnids. The models indicated that adverse growth effects would become apparent at PAH levels approximately 100 times the median environmental concentrations. The models further predicted that daphnid growth rates would be progressively reduced at mixture levels between 100 and 1000 times the median environmental concentrations.

Direct evaluation of the effects of this PAH mixture on daphnid growth rates supported the concentration addition model predictions (Fig. 2A). The coefficient of determination (r^2) for the toxic equivalency model

Table 2
Parameters used to model the toxicity of PAH mixtures

PAH	EC50 (μg/L)	ρ
Pyrene	72.7 ± 7.8	2.41 ± 0.64
Phenanthrene	349 ± 19	1.46 ± 0.12
Fluoranthene	194 ± 11	1.85 ± 0.17
Naphthalene	4610 ± 820	1.55 ± 0.29

Values were determined from individual toxicity tests and are represented as the parameter estimate plus or minus the error of that estimate. Data from individual PAH toxicity assessments were fit with Eq. (4) to yield the parameters.

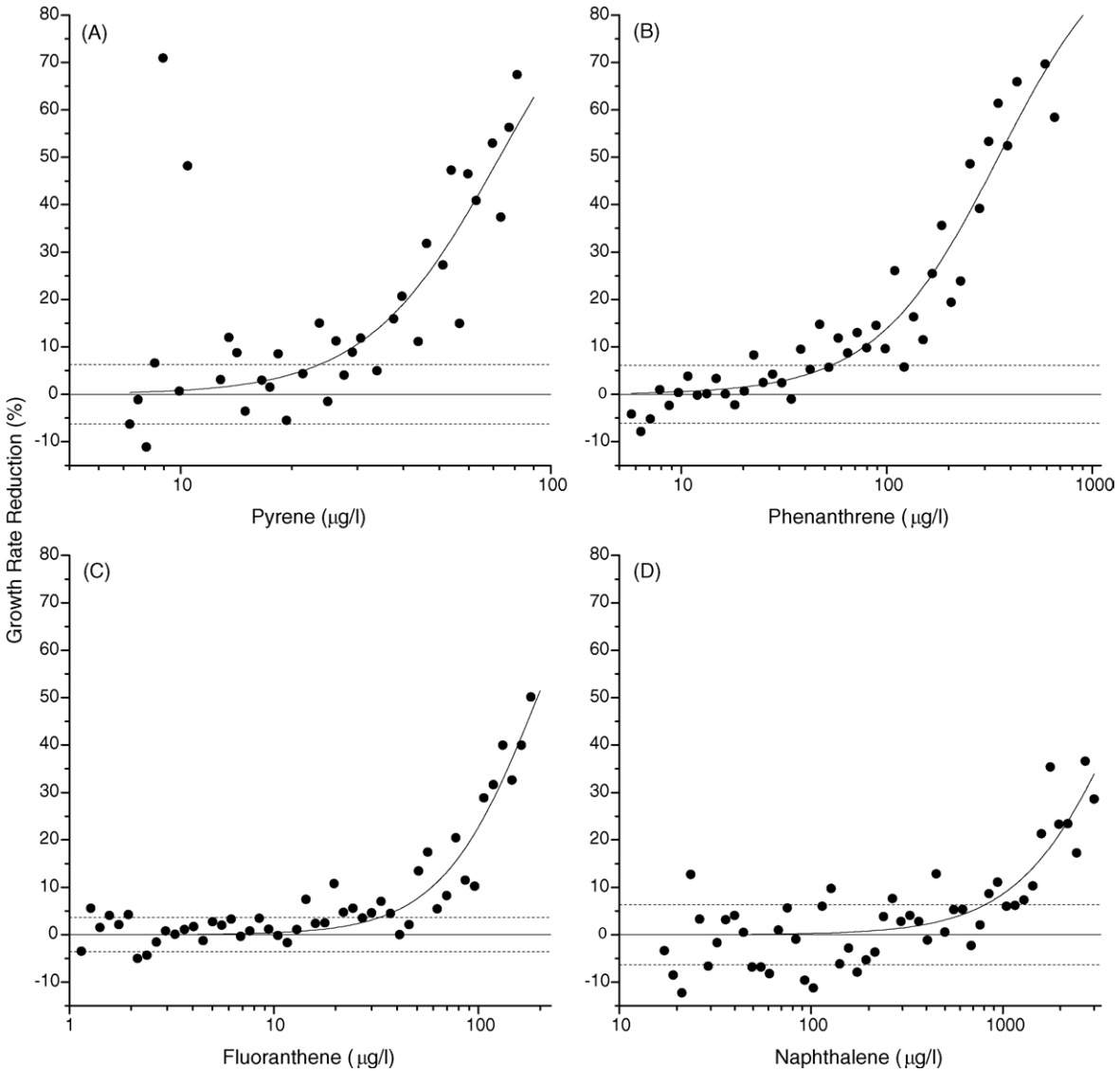

Fig. 1. Reductions in growth rate of daphnids exposed to concentrations of individual PAHs. Each data point represents the percentage growth rate reduction of a single daphnid when compared to the mean growth rate of 10 control daphnids. The horizontal solid line depicts the performance of the control organisms and the horizontal dashed lines represent the standard deviation of the control response. The curve fit to the data was derived using Eq. (3). (A) Pyrene; (B) phenanthrene; (C) fluoranthene and (D) naphthalene.

was 0.73, indicating that 73% of the variation in growth rate reduction measured among exposed daphnids was accounted for by the model. The general concentration addition model yielded predictions comparable to those observed using the toxic equivalency approach ($r^2 = 0.77$).

PAH mixtures were next evaluated in which the ratio of the individual constituents was equivalent to the ratio of the chemicals when present at their maximum reported environmental levels. Both the toxic equivalency and general concentration addition models indicated that PAHs present at their maximum environ-

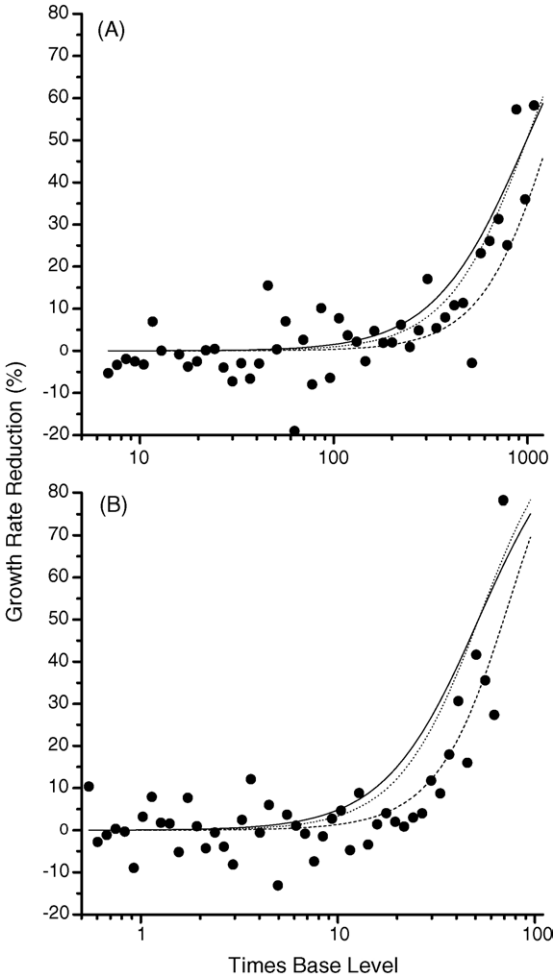

Fig. 2. Reductions in growth rates of daphnids exposed to environmentally relevant PAH mixtures. Each data point represents the percentage growth rate reduction of a single daphnid when compared to the mean growth rate of 10 control daphnids. The solid lines represent the model prediction of the response of the organisms to the mixtures using the toxic equivalency model. The dashed lines represent the model predictions using the independent joint action approach. The dotted lines represent the general concentration addition model. (A) Base level is the set of PAH concentrations reported as median detected concentrations in a survey of US freshwaters (Kolpin et al., 2002). (B) Base level is the set of PAH concentrations reported as the maximum detected concentrations in the same survey.

mental levels (base level = 1, Fig. 2B) would elicit no adverse effect on growth of daphnids; however, adverse effects on growth rate would become evident at exposure levels approximating five times reported maximum levels (Table 1). The models further predicted that daphnid growth rates would be progressively reduced with increasing mixture exposure levels greater than this threshold level.

Direct evaluation of the effects of this PAH mixture on daphnid growth rates indicated that the concentration addition models (Fig. 2B, toxic equivalency, solid line; general concentration addition, dotted line) overestimated the toxicity of this mixture (Fig. 2B). The threshold response level for the mixture was approximately three times (∼15× base level) that estimated by the concentration addition models. Further, the low coefficient of determination (r^2) for the toxic equivalency (0.46) and general concentration addition (0.53) models indicated a significant reduction in predictive capability of these models using this data set.

Both mixture formulations were re-evaluated using the independent joint action (Eq. (3)) model in an effort to discern whether the PAH mixtures better conform to this approach. When using the mixture formulation consisting of a ratio of PAHs when present at median environmental levels, the performance of the independent joint action model was comparable to that of the concentration addition models ($r^2 = 0.71$ for independent joint action model, $r^2 = 0.73$ for toxic equivalency model and $r^2 = 0.77$ for the general concentration addition model, Fig. 2A). The independent joint action model appreciably improved the predictive modeling for the mixture consisting of a ratio of PAHs representing maximum environmental levels ($r^2 = 0.77$ for independent joint action model, $r^2 = 0.46$ for toxic equivalency model and $r^2 = 0.53$ for the general concentration addition model, Fig. 2B).

Results from these mixtures experiments suggested that the combined toxicity of the PAHs better conforms to a model for independent joint action as opposed to concentration addition. In an attempt to confirm that the toxicity of PAH mixtures better conforms to a model of independent joint action, the effect of a PAH mixture on growth rate reduction was modeled and experimentally determined when the base level of the mixture contained each PAH at its EC05 level. Fifty dilutions or fortifications of this mixture were tested directly and modeled according to concentration addition (both toxic equivalency model and general concentration addition model) and independent joint action (Fig. 3A). The independent joint action model was appreciably more accurate in modeling the toxi-

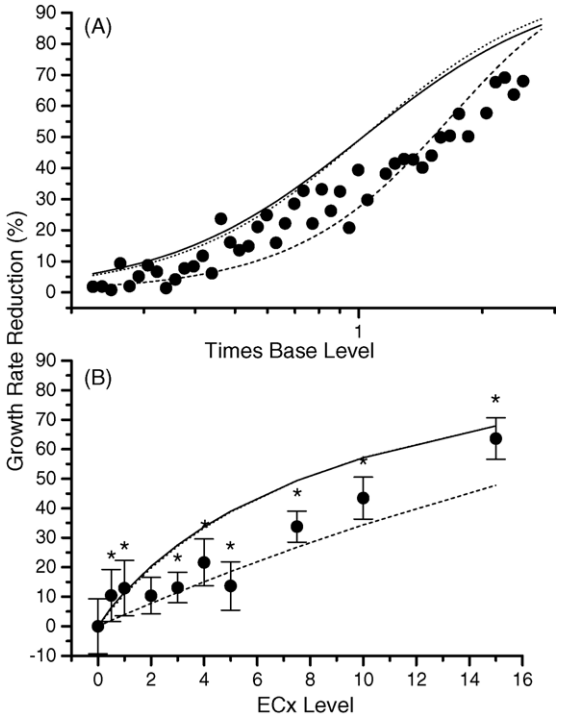

Fig. 3. Modeled and measured growth rates of daphnids exposed to equi-toxic PAH mixtures. Growth rate reduction represents the percentage growth rate reduction when compared to the mean growth rate of 10 control daphnids. The solid lines represent the prediction of the response of the organisms to the mixtures using the toxic equivalency model. The dashed lines represent the model prediction using the independent joint action approach. The dotted lines represent the general concentration addition model. (A) Each data point represents the percentage growth rate reduction of a single daphnid. Base level represents the mixture of PAHs with each constituent present at its EC05 level. (B) Each data point represents the mean and standard deviation growth rate reduction of ten daphnids exposed to various equi-toxic mixtures of the PAHs. *X*-axis represents effect concentration (i.e. EC02, EC04, etc.) at which each constituent of the mixture is present. An asterisk denotes a significant difference from the control response ($\alpha = 0.05$, Dunnett's *t*-test).

city of the mixture than either concentration addition methods ($r^2 = 0.87$ for independent joint action model, $r^2 = 0.59$ for toxic equivalency model and $r^2 = 0.54$ for the general concentration addition model, Fig. 3A).

Finally, seven equi-toxic mixtures of the PAHs ranging from a mixture of each chemical at its EC005 level to its EC15 level were modeled and experimentally evaluated for toxicity (Fig. 3B). Again, the independent joint action model was much more accurate ($r^2 = 0.65$) at modeling the effects of the mixtures on daphnid growth rate as compared to the toxic equivalency ($r^2 = 0.32$) or general concentration addition ($r^2 = 0.33$) models.

4. Discussion

Based on an assumption of common mode of action among the four PAHs evaluated, we hypothesized that a concentration addition model would accurately predict the effects of exposure to mixtures of these chemicals on daphnid growth rates. In general, both concentration addition models over-predicted the responses when compared to experimental results. The alternative model of independent joint action, that does not assume a common mode of action, more accurately predicted the toxicity of the mixtures. Despite this increase in performance, predictions between all models were not dramatically different, and all models would provide a reasonable prediction of the toxicity of mixtures of these PAHs.

One explanation for the discrepancy between the responses predicted by the toxic equivalency approach and the experimental results is a violation of the assumption of a common mode of action among the four PAHs. PAHs can elicit toxicity through several mechanisms. These include non-polar narcosis, adduct formation, the generation of reactive oxygen radicals and hormonal disturbance (Arfsten et al., 1996; van Brummelen et al., 1998; Teles et al., 2005). Modeling efforts may have been slightly compromised by different PAHs eliciting effects on growth by different mechanisms of action. For example, fluoranthene is a weak ecdysteroid receptor antagonist in *Drosophila* B_{11} cells (Dinan et al., 2001). Antagonism of the ecdysteroid receptor can interfere with molt frequency (Mu and LeBlanc, 2002) which can negatively impact growth rate, as was measured in the present study.

The PAH pyrene has been shown to modify the ability of ecdysone to activate the ecdysone receptor in an EcR reporter gene construct and in ecdysteroid-responsive C1.8 cells (Oberdorster et al., 1999). The authors concluded that this effect was independent of any interaction of pyrene with the EcR. Based upon these observations, pyrene may perturb ecdysteroid signaling and impact growth rates though a mechanism

distinct from that of fluoranthene. We noted that both pyrene and phenanthrene decreased molt frequency of the daphnids; whereas, naphthalene and fluoranthrene did not elicit this effect. Thus, pyrene and phenanthrene may have elicited effects on growth rates through a molting-dependent pathway that was distinct from the mode of action of naphthalene and fluoranthene. A multiplicity of targets by which the PAHs affected growth could explain the increased precision of the independent joint action model over the concentration addition model in defining the combined effects of the PAHs.

A toxic units approach (akin to the general concentration addition approach used in this study) has been used to predict the acute toxicity of sediment-associated PAH mixtures to aquatic organisms with varying levels of success. Concentration addition modeling using toxic units under-predicted the toxicity of PAH mixtures to three invertebrate species leading the authors to conclude that the PAHs acted synergistically (Verrhiest et al., 2001). Swartz et al. (1995) reported no significant differences between observed toxicity of PAH mixtures to amphipods and modeled toxicity using an approach incorporating toxic units. However, the same model slightly, but significantly, overestimated toxicity associated with a mixture of photoactivated PAHs (Swartz et al., 1997). In all cases and consistent with our results, the reported deviations from concentration additivity were not great and the concentration addition models provided a reasonable description of the response of organisms to the PAH mixtures.

Munoz and Tarazona (1993) evaluated the acute toxicity of equi-toxic formulations of four PAHs to *D. magna*. These formulations consisted of phenanthrene and naphthalene, as used in our study, along with anthracene and acenaphthene. Three formulations were assessed and all were slightly less toxic than predicted using a toxic unit approach. These observations were consistent with the PAH mixtures assessed in the present study (Fig. 3) and provide additional support to the contention that concentration addition models slightly overestimate the toxicity of PAH mixtures.

In recent years, significant attention has been directed towards the premise that mixtures, in which individual constituents are present at levels that elicit low or no response, may elicit significant toxicity in combination (Kortenkamp and Altenburger, 1998; Helge et al., 2002; Silva et al., 2002). In the absence of chemical interactions that can modify toxicity (i.e. Mu and LeBlanc, 2004; Rider and LeBlanc, in press), mixtures of chemicals that are individually present at or below their individual threshold effect levels, can elicit significant toxicity if the chemicals are like acting and conform to a model of concentration addition. However, significant toxicity of the mixture would not be expected if the constituents elicit toxicity via different mechanisms and conform to a model for independent joint action (note that model predictions of effects are always greater using the concentration addition model as compared to the independent joint action model in Figs. 2 and 3). Results from the present study demonstrate that combined toxicity for these PAHs, when constituents were present at concentrations that individually elicited little toxicity (i.e. EC02, EC03, etc. levels in Fig. 3B) elicited toxicity no more than that expected by the individual constituents. Thus, this mixture would not be expected to elicit significant toxicity when individual constituents are present at sub-effect levels.

In the present study, PAH mixture effects were modeled and experimentally evaluated at concentrations that were relevant to ambient freshwater environmental levels. While the PAH formulations did adversely impact growth rates of the daphnids, these effects were predicted to occur and were measured at mixture levels $\geq$100 times greater than reported median environmental levels of the four compounds (Kolpin et al., 2002). A safety margin of $\sim$5 existed for the mixture formulation representative of maximum reported levels of the four compounds. These results suggest that typical ambient levels of these four common PAHs in combination would pose little direct risk to daphnid populations due to additive or synergistic interactions among the constituents. PAHs, however, are known to undergo photoactivation, which could result in increased toxicity beyond those measured in the present study under some ambient conditions.

Acknowledgements

This work was supported by US Environmental Protection Agency STAR grant R829358 and US Department of Energy cooperative agreement DE-FC26-OONT40843.

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